

U.S.S.N.: 09/403,690  
Attorney Docket No: 20239-0706-US  
Client Docket No: BN 25 PCT-US

## Appendix II

### Currently Pending Claims

21. (Amended) A method for detection and differentiation of pathogenic enterobacteria in a sample, said method comprising:

isolating nucleic acid from said sample;

adding a set of oligonucleotide primer pairs to said sample, wherein said set of oligonucleotide primers comprises at least five oligonucleotide primer pairs, wherein at least one primer pair is capable of specifically amplifying a DNA sequence to produce an amplified product of a virulence factor/toxin gene characteristic for each one of an enterotoxigenic, enteroaggregative, enteroinvasive, enteropathogenic or enterohemorrhagic pathogenic *E. coli* strains;

subjecting said sample and said set of primer pairs to an amplification process; and

detecting the presence of at least one amplified product, wherein the presence of at least one amplified product indicates the presence of at least one pathogenic enterobacteria strain in said sample.

22. (Amended) The method according to claim 21, wherein the set of oligonucleotide primer pairs comprises primer pairs selected from the group consisting of:

at least one primer pair that hybridizes to a gene encoding heat labile toxin or a gene encoding heat stabile toxin for amplification of a DNA sequence characteristic for enterotoxigenic *E. coli*;

at least one primer pair that hybridizes to a gene encoding heat stabile toxin or to a pCDVD432 plasmid for amplification of a DNA sequence characteristic for enteroaggregative *E. coli*;

at least one primer pair that hybridizes to a *inv*-plasmid for amplification of a DNA sequence characteristic for enteroinvasive *E. coli*;

at least one primer pair that hybridizes to a *EAF* plasmid, or an *eae* gene for amplification of a DNA sequence characteristic for enteropathogenic *E. coli*; and

at least one primer pair that hybridizes to the genes encoding shiga-like toxin *sltI* or *sltII* for amplification of a DNA sequence characteristic for enterohemorrhagic *E. coli*.

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23. (Reiterated) The method according to claim 22, wherein the oligonucleotide primer pair that hybridizes to the gene encoding heat labile toxin characteristic for enterotoxigenic *E. coli* is

LT-1: 5' GCG TTA CTA TCC TCT CTA TGT G 3' (SEQ ID NO: 1) and LT-2: 5' AGT TTT CCA TAC TGA TTG CCG C 3' (SEQ ID NO: 2); and

the oligonucleotide primer pair that hybridizes to the gene encoding heat stabile toxin characteristic for enterotoxigenic *E. coli* is

ST-1: 5' TCC CTC AGG ATG CTA AAC CAG 3' (SEQ ID NO: 3) and ST-2a: 5' TCG ATT TAT TCA ACA AAG CAA C 3' (SEQ ID NO: 4); and

the oligonucleotide primer pair that hybridizes to the gene encoding heat stabile toxin characteristic for enteroaggregative *E. coli* is

EASTI-1: 5' AAC TGC TGG GTA TGT GGC TGG 3' (SEQ ID NO: 5) and EASTI-2: 5' TGC TGA CCT GCC TCT TCC ATG 3' (SEQ ID NO: 6); and

the oligonucleotide primer pair which hybridizes to the pCVD432 plasmid is

EA-1: 5' CTG GCG AAA GAC TGT ATC ATT G 3' (SEQ ID NO: 7) and EA-2: 5' TAA TGT ATA GAA ATC CGC TGT T 3' (SEQ ID NO: 8); and

the oligonucleotide primer pair which hybridizes to the *inv*-plasmid is

EI-1: 5' TTT CTG GAT GGT ATG GTG AGG 3' (SEQ ID NO: 9) and EI-2: 5' CTT GAA CAT AAG GAA ATA AAC 3' (SEQ ID NO: 10); and

the oligonucleotide primer pair which hybridizes to the EAF plasmid is

EP-1: 5' CAG GGT AAA AGA AAG ATG ATA AG 3' (SEQ ID NO: 11) and EP-2: 5' AAT ATG GGG ACC ATG TAT TAT C 3' (SEQ ID NO: 12); and

the oligonucleotide primer pair which hybridizes to the *eae* gene is

EPeh-1: 5' CCC GCA CCC GGC ACA AGC ATA AG 3' (SEQ ID NO: 13) and EPeh-2: 5' AGT CTC GCC AGT ATT CGC CAC C 3' (SEQ ID NO: 14); and

the oligonucleotide primer pair which hybridizes to the gene encoding shiga-like toxin SltI is

SHI-1: 5' ATG AAA AAA ACA TTA TTA ATA GC 3' (SEQ ID NO: 15) and SHI-2: 5' TCA CYG AGC TAT TCT GAG TCA AGC 3' (SEQ ID NO: 16); and

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the oligonucleotide primer pair which hybridizes to the gene encoding shiga-like toxin SltII is

SltII-1: 5' ATG AAG AAG ATR WTT RTD GCR CYT TTA TTY G 3' (SEQ ID NO:17)  
and SltII-2: 5' TCA GTC ATW ATT AAA CTK CAC YTS RGC AAA KCC 3' (SEQ ID NO:18),

wherein W is A/T, R is A/G, D is A/G/T, Y is C/T and K is G/T.

24. (Amended) The method according to claim 21, wherein detecting the presence of at least one amplified product is performed using at least one oligonucleotide probe capable of hybridizing to the amplified product wherein said oligonucleotide probe is labeled at the 5' end with a fluorescent reporter dye and at the 3' end with a fluorescent quencher dye and is susceptible to 5'-3' exonuclease degradation by a polymerase, and wherein said amplification process uses a polymerase having 5'-3' exonuclease degradation activity.

25. (Cancelled)

26. (Amended) The method according to claim 24 wherein the labeled oligonucleotide probe is selected from the group consisting of:

a labeled oligonucleotide probe specific for the detection of a heat labile toxin gene characteristic for enterotoxigenic E. Coli;

a labeled oligonucleotide probe specific for the detection of a heat stabile toxin gene characteristic for enterotoxigenic E. Coli;

a labeled oligonucleotide probe specific for the detection of a heat stabile toxin gene characteristic for enteroaggregative E. Coli;

a labeled oligonucleotide probe specific for the detection of a pCVD432 plasmid;

a labeled oligonucleotide probe specific for the detection of a inv-plasmid;

a labeled oligonucleotide probe specific for the detection of a EAF-plasmid;

a labeled oligonucleotide probe specific for the detection of a eae gene;

a labeled oligonucleotide probe specific for the detection of a shiga-like toxin SltI gene; and

a labeled oligonucleotide probe specific for the detection of a shiga-like toxin SltII gene.

27. (Reiterated) The method according to claim 26, wherein the labeled oligonucleotide probe for the detection of heat labile toxin gene characteristic for enterotoxigenic E. coli is

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5' AGC TCC CCA GTC TAT TAC AGA ACT ATG 3' (SEQ ID NO: 19),

the labeled oligonucleotide probe for the detection of heat stabile toxin gene characteristic for enterotoxigenic *E. coli* is

5' ACA TAC GTT ACA GAC ATA ATC AGA ATC AG 3' (SEQ ID NO: 20);

the labeled oligonucleotide probe for the detection of heat stabile toxin gene characteristic for enteroaggregative *E. coli* is

5' ATG AAG GGG CGA AGT TCT GGC TCA ATG TGC 3' (SEQ ID NO: 21);

the labeled oligonucleotide probe for the detection of pCVD432 plasmid is

3' CTC TTT TAA CTT ATG ATA TGT AAT GTC TGG 3' (SEQ ID NO: 22);

the labeled oligonucleotide probe for the detection of the *inv*-plasmid is

5' CAA AAA CAG AAG AAC CTA TGT CTA CCT 3' (SEQ ID NO: 23)

the labeled oligonucleotide probe for the detection of the *EAF*-plasmid is

5' CTT GGA GTG ATC GAA CGG GAT CCA AAT 3' (SEQ ID NO: 24);

the labeled oligonucleotide probe for the detection of the *eae* gene is

5' TAA ACG GGT ATT ATC AAC AGA AAA ATC C 3' (SEQ ID NO: 25);

the labeled oligonucleotide probe for the detection of shiga-like toxin *StxI* gene is

5' TCG CTG AAT CCC CCT CCA TTA TGA CAG GCA 3' (SEQ ID NO: 26);

and the labeled oligonucleotide probe for the detection of shiga-like toxin *StxII* gene is

5' CAG GTA CTG GAT TTG ATT GTG ACA GTC ATT 3' (SEQ ID NO: 27).

28. (Reiterated) The method according to claim 24 wherein the fluorescent reporter dye is 6-carboxy-fluorescein, tetrachloro-6-carboxy-fluorescein, or hexachloro-6-carboxy-fluorescein, and the fluorescent quencher dye is 6-carboxytetramethyl-rhodamine.

29. (Amended) The method according to claim 21 wherein the amplification process comprises 35 PCR cycles at a  $MgCl_2$  concentration of 5.2 mM, an annealing temperature of 55 °C and an extension temperature of 65 °C.

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30. (Amended) A set of oligonucleotide primer pairs useful for polymerase chain reaction (PCR) amplification of DNA of pathogenic enterobacteria allowing detection and differentiation of pathogenic enterobacteria in a sample wherein following amplification the presence of at least one amplified product indicates the presence of at least one pathogenic enterobacteria strain in said sample, wherein said set comprises at least five primer pairs, wherein each primer pair specifically amplifies a DNA sequence of a virulence factor/toxin gene characteristic for one each of the subgroups of the pathogenic *E. coli* strains, said subgroups comprising enterotoxigenic, enteroaggregative, enteroinvasive, enteropathogenic and enterohemorrhagic *E. coli* strains and wherein for amplification of each subgroup at least one oligonucleotide primer pair is included in said set of oligonucleotide primer pairs.

31. (Amended) The set of primer pairs according to claim 30 comprising

a primer pair that hybridizes to a gene encoding heat labile toxin, or to a gene encoding heat stabile toxin of enterotoxigenic *E. coli*;

a primer pair that hybridizes to a gene encoding heat stabile toxin or to a pCVD432 plasmid of enteroaggregative *E. coli*;

a primer pair that hybridizes to a *inv*-plasmid of enteroinvasive *E. coli*;

a primer pair that hybridizes to a EAF plasmid, or a *eae* gene of enteropathogenic *E. coli*; and

a primer pair that hybridizes to a gene encoding shiga-like toxin *sltI* or *sltII* of enterohemorrhagic *E. coli*.

32. (Reiterated) The set of primer pairs according to claim 31 wherein

the primer pair which hybridizes to the gene encoding heat labile toxin of enterotoxigenic *E. coli* is

LT-1: 5' GCG TTA CTA TCC TCT CTA TGT G 3' (SEQ ID NO: 1) and

LT-2: 5' AGT TTT CCA TAC TGA TTG CCG C 3' (SEQ ID NO: 2);

the primer pair which hybridizes to the gene encoding heat stabile toxin of enterotoxigenic *E. coli* is

ST-1: 5' TCC CTC AGG ATG CTA AAC CAG 3' (SEQ ID NO: 3) and

ST-2a: 5' TCG ATT TAT TCA ACA AAG CAA C 3' (SEQ ID NO: 4);

the primer pair which hybridizes to the gene encoding heat stabile toxin of enteroaggregative *E. coli* is

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EASTI-1: 5' AAC TGC TGG GTA TGT GGC TGG 3' (SEQ ID NO: 5) and

EASTI-2: 5' TGC TGA CCT GCC TCT TCC ATG 3' (SEQ ID NO: 6);

the primer pair which hybridizes to the pCVD432 plasmid is

EA-1: 5' CTG GCG AAA GAC TGT ATC ATT G 3' (SEQ ID NO: 7) and

EA-2: 5' TAA TGT ATA GAA ATC CGC TGT T 3' (SEQ ID NO: 8);

the primer pair which hybridizes to the inv-plasmid is

EI-1: 5' TTT CTG GAT GGT ATG GTG AGG 3' (SEQ ID NO: 9) and

EI-2: 5' CTT GAA CAT AAG GAA ATA AAC 3' (SEQ ID NO: 10);

the primer pair which hybridizes to the EAF plasmid is

EP-1: 5' CAG GGT AAA AGA AAG ATG ATA AG 3' (SEQ ID NO: 11) and

EP-2: 5' AAT ATG GGG ACC ATG TAT TAT C 3' (SEQ ID NO: 12);

the primer pair which hybridizes to the eae gene is

EPeh-1: 5' CCC GGA CCC GGC ACA AGC ATA AG 3' (SEQ ID NO: 13) and

EPeh-2: 5' AGT CTC GCC AGT ATT CGC CAC C 3' (SEQ ID NO: 14);

the primer pair which hybridizes to the shiga-like toxin sttI gene is

SttI-1: 5' ATG AAA AAA ACA TTA TTA ATA GC 3' (SEQ ID NO: 15) and

SttI-2: 5' TCA CYG AGC TAT TCT GAG TCA AGC 3' (SEQ ID NO: 16); and

the primer pair which hybridizes to the shiga-like toxin sttII is

SttII-1: 5' ATG AAG AAG ATR WTT RTD GCR GYT TTA TTY G 3' (SEQ ID NO: 17)  
and

SttII-2: 5' TCA GTC ATW ATT AAA CTK CAC YTS RGC AAA KCC 3' (SEQ ID NO:  
18)

wherein W is A/T, R is A/G, D is A/G/T, Y is C/T and K is G/T.

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33. (Amended) A set of labeled oligonucleotide probes useful for detection and differentiation of pathogenic enterobacteria in a sample by Real Time-PCR, each probe specifically binding a sequence of a virulence factor/toxin genes characteristic of one of the subgroups of pathogenic *E. coli* strains comprising enterotoxigenic, enteroaggregative, enteroinvasive, enteropathogenic and enterohemorrhagic *E. coli* strains, wherein said oligonucleotide probes are labeled at the 5' end with a fluorescent reporter dye and at the 3' end with a fluorescent quencher dye and are susceptible to 5'-3' exonuclease degradation by a polymerase, and wherein for detection and differentiation of each subgroup at least one probe is included in the set of oligonucleotide probes.

34. (Amended) The set of probes according to claim 33 comprising:

a labeled oligonucleotide probe specific for the detection of heat labile toxin gene characteristic for enterotoxigenic *E. Coli* or a labeled oligonucleotide probe specific for the detection of heat stabile toxin gene characteristic for enterotoxigenic *E. Coli*;

a labeled oligonucleotide probe specific for the detection of heat stabile toxin gene characteristic for enteroaggregative *E. Coli* or a labeled oligonucleotide probe specific for the detection of pCVD432 plasmid;

a labeled oligonucleotide probe specific for the detection of the *inv*-plasmid;

a labeled oligonucleotide probe specific for the detection of the *EAF*-plasmid or a labeled oligonucleotide probe specific for the detection of the *eae* gene; and

a labeled oligonucleotide probe specific for the detection of shiga-like toxin *StxI* gene or a labeled oligonucleotide probe specific for the detection of shiga-like toxin *StxII* gene.

35. (Reiterated) The set of probes according to claim 34, wherein

the labeled oligonucleotide probe for the detection of heat labile toxin gene characteristic for enterotoxigenic *E. coli* is

5' AGC TCC CCA GTC TAT TAC AGA ACT ATG 3' (SEQ ID NO: 19);

the labeled oligonucleotide probe for the detection of heat stabile toxin gene characteristic for enterotoxigenic *E. coli* is

5' ACA TAC GTT ACA GAC ATA ATC AGA ATC AG 3' (SEQ ID NO: 20);

the labeled oligonucleotide probe for the detection of heat stabile toxin gene characteristic for enteroaggregative *E. coli* is

5' ATG AAG GGG CGA AGT TCT GGC TCA ATG TGC 3' (SEQ ID NO: 21);

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the labeled oligonucleotide probe for the detection of pCVD432 plasmid is

5' CTC TTT TAA CTT ATG ATA TGT AAT GTC TGG 3' (SEQ ID NO: 22);

the labeled oligonucleotide probe for the detection of the inv-plasmid is

5' CAA AAA CAG AAG AAC CTA TGT CTA CCT 3' (SEQ ID NO: 23)

the labeled oligonucleotide probe for the detection of the EAF-plasmid is

5' CTT GGA GTG ATC GAA CGG GAT CCA AAT 3' (SEQ ID NO: 24)

the labeled oligonucleotide probe for the detection of the eae gene is

5' TAA ACG GGT ATT ATC AAC AGA AAA ATC C 3' (SEQ ID NO: 25)

the labeled oligonucleotide probe for the detection of shiga-like toxin StI gene is

5' TCG CTG AAT CCC CCT CCA TTA TGA CAG GCA 3' (SEQ ID NO: 26);

the labeled oligonucleotide probe for the detection of shiga-like toxin StII gene is

5' CAG GTA CTG GAT TTG ATT GTG ACA GTC ATT 3' (SEQ ID NO: 27).

36. (Amended) A set of oligonucleotide primer pairs and a set of oligonucleotide primer probes useful for diagnosing an enterobacteria infection in samples derived from a living animal body including a human, by Real time PCR method, wherein said sets of oligonucleotide primer pairs and oligonucleotide primer probes allow detection and differentiation of pathogenic enterobacteria in a sample, wherein said set of oligonucleotide primer pairs comprises at least five primer pairs and at least one primer pair specifically amplifies a DNA sequence of a virulence factor/toxin gene characteristic for each of the subgroups of the pathogenic *E. coli* strains, said subgroups comprising enterotoxigenic, enteroaggregative, enteroinvasive, enteropathogenic and enterohemorrhagic *E. coli* strains and, wherein said set of oligonucleotide probes comprises at least one oligonucleotide probe, each oligonucleotide probe specifically binding a sequence of a virulence factor/toxin genes characteristic of one of the subgroups of pathogenic *E. coli* strains, said subgroups comprising enterotoxigenic, enteroaggregative, enteroinvasive, enteropathogenic and enterohemorrhagic *E. coli* strains by real time PCR.

37. (Reiterated) The method of claim 21, wherein said method is used to diagnose an enterobacteria infection in a sample derived from a living animal body.

38. (Amended) The method of claim 37, wherein said sample is derived from a human.



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**39. (Reiterated) The method of claim 21, wherein said method is used to detect enterobacteria contamination of a consumable.**

**40. (Amended) The method of claim 39, wherein said consumable is selected from the group consisting of meat, milk and vegetable.**